Phylogenic study on *Ephedra* plants related to *Ephedra sinica* by sequence analysis of the internal transcribed spacer 2 region (ITS2) in nuclear ribosomal DNA

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Abstract

In East Asia, the aerial part of *Ephedra* plants has been staple crude drug from ancient times. Among them, crude drug derived from *E. sinica* is considered to be the most widely distributed in both Chinese and Japanese markets. *E. sinica* has herbaceous appearance and belongs to one of 3 phyletic groups of Chinese *Ephedra* plants. In morphological respect, *E. sinica* is very similar to *E. dahurica*, and the Central Asian species *E. pseudodistachya*, On the other hand, delimitation of *E. lomatolepis* as well as from the European/West Asian *E. distachya* is controversial. Aiming to develop new *Ephedra* plant resources and evaluate them as crude drug, the genetic relationship was examined using ITS 2 sequences of an extended sampling from West Europe to East Asia. As the result, following findings were made: (1) specimens of E. sinica from China and Mongolia were almost identical to those of *E. dahurica* from southern Buryatia and Chita. (2) Each of *E. pseudodistachya* and *E. lomatolepis* specimens from West to Central Asia formed a loose cluster with *E. sinica* and *E. dahurica*. (3) Specimens of *E. distachya* from West Asia to Europe grouped together. The results confirmed the result obtained from ITS1 analysis as well as hypothesis based on morphology that *E. sinica* and *E. dahurica* should be merged under the same name.

Key words : *Ephedra*, phylogeny, nrDNA, Eurasia

Introduction

The aerial part of *Ephedra* plants has been used as a staple crude drug in Far Eastern countries including China and Japan since ancient times. In Japan, Ephedra is prescribed in commonly-used prescriptions such as Pueraria combination (葛根湯), Ephedra combination (麻黄湯) and Ephedra, Pinellia and Schisandra combination (小青龍湯)[1]. More than 50 species of *Ephedra* plants distribute worldwide and were reported to contain variety of alkaloids[2]. Among them, *Ephedra sinica* is currently the most commonly used crude drug in the Japanese and Chinese market[3]. *E. sinica* is reported to grow mainly in the northeastern part of China, while there are some *Ephedra* plants, whose morphology are reported to be similar to *E. sinica*, growing in the other part of Eurasia, namely, *E. dahurica, E. distachya, E lomatolepis* and *E. pseudodistachya*. *E. dahurica* grows in the eastern part of Siberia, and E. distachya in the Mediterranean Coast region, and *E. lomatolepis* and *E. pseudodistachya* in the Central Asia. We intended to study their phylogenic relationship by molecular biological method. Internal transcribed spacers (ITSs) are spacer sequences contained in transcription unit of ribosomal RNAs (18S RNA, 5.8S RNA and 28S RNA). The unit is repeated thousands times in genome. ITSs are often used for phylogenic analysis of closely related taxa of plants as well as animals, since DNA sequence mutation accumulates there more frequently than coding regions. We studied ITS1 of *Ephedra* plants relating *E. sinica*, and found that *E. sinica* and *E. dahurica* had almost identical sequence

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and were considered the same taxon[4]. We also found that *E. sinica/E. dahurica* and *E. distachya* were not monophyletic. In present paper, we report the result of phylogenic study on these *Ephedra* plants relating *Ehedra sinica* using ITS 2.

Materials and Methods

Materials

The specimens used are listed in Table 1 and are deposited in the Herbarium either of Faculty of Pharmaceutical, Kanazawa University or Kassel University, respectively.

DNA preparation

The plant stem from each specimen was cut into 2 mm-pieces, frozen in liquid nitrogen and ground into powder. Using a DNeasy Plant Mini Kit (Qiagen), the DNA was extracted according to the manufacturer's protocol.

PCR amplification

Total DNA was used as a template for amplifying the ITS2 regions by PCR. The primers were designed based on 5.8S and 26S nuclear ribosomal DNA from Genbank[5]. The primer set of 5.8S-F (GAA CGT AGC GAA ATG CGA TA) /Eph-1R (GTA AGT TTC TCT TCC TCC GC) was used for the amplification. Standard PCR was performed in 25 µl of reaction mixture containing 2.5 µl of 10 × PCR buffer for KOD-Plus, 0.2 mM each of dNTP, 1 mM $MgSO_4$, 0.4 µM of each primer, approximately 100 ng of DNA sample and 0.5 units of KOD-Plus DNA polymerase (Toyobo). PCR was carried out as follows: hot start at 94℃ for 2 min, 30 cycles of denaturation at 94℃ for 15 sec, annealing at 55℃ for 30 sec and elongation at 68℃ for 45 sec, and a final elongation at 68℃ for 5 min. Three µl of the PCR product was used for agarose gel electrophoresis and the remaining product was purified using the QIA quick PCR Purification Kit (Qiagen).

Sequencing

The purified PCR product was subjected to direct sequencing using a DTCS Quick Start Master Mix Kit (Beckman Coulter) and with a CEQ Genetic Analyzing System (Beckman Coulter). The primers, Eph-1R and 5.8S F, were used for priming the sequencing reaction. The DNA sequences were aligned using `DNASIS` version 3.0 software (Hitachi). The sequence data were submitted to GenBank, and the accession numbers are listed in Table 1.

Result and Discussion

As listed in Table 1, the specimens were collected from the Far East region of Russia to the Mediterranean region (Fig. 1). The genetic relationship was examined

Fig 1 Map of collecting sites

Table 1 The list of samples

Specimen ID	Morphological identification	GenBank Acc No.	Voucher	location
Brt1	*E. dahurica*	JQ726575	F. 33.121	Russia, Buryatia, Gusinoozersk
Brt2	*E. dahurica*	GU290483	H. Freitag 33119	Russia: Buryatia c.95 km SW Ulan-Ude,
Brt3	*E.dahurica*	GU186923	KANP 060804337	Russia, Buryatia, suburb of UlanUde,
Cht	*E. dahurica*	GU290481.	F. 33.130	Russia, Chita, Daurya Biosphere reserve
Fom	*E. foeminea*	GU290484	Kephallonia, old fortress near Peratataleg. et det. H. Freitag 19.09.1989, no 19.807	Greece, Kephallonia
Itl	*E. distachya ssp. helvetica*	JQ726579	F. 26.677/78	S Tyrol, Vintschgau, Schlanders
Kzh1	*E. distachya*	JQ726577	F. 26.506	Kazakhstan, Novokazalinsk
Kzh2	*E. lomatolepis*	JQ726585	H. Freitag, S.Rilke 26507	Kazakhstan, Kzyl-Orda dist.
Kzh3	*E. pseudodistachya*	JQ726587	H. Freitag 27000	Kazakhstan, Alimtau
Kzh4	*E. lomatolepis*	JQ726586	H. Freitag, S.Rilke 26343a	Kazakhstan, Dzhambul dist.
Kzh5	*E. lomatolepis*	GU290482	Freitag 26.355	Kazakhstan, Dzhambul dist.
Kzh6	*E.prezewalskii?*	JQ726588	H. Freitag, S. Rilke 26084	SE Kasachstan,Alma-Ata distr
Mng1	*E. pseudodistachya*	JQ726592	Miehe 96-033-03	Mongolia, Altai Aimak
Mng2	*E. sinica*	GU186922	KANP 20531001	Lun, Tov dist. Mongolia
Pak	*E. procera*	JQ726580	H. Freitag 18.875	Pakistan, N Baluchisan
Sws6	*E.distachya*	GU186924	KANP 08080801	Sion Castle, Sion, Switzerland
Tbt1	*E.likiangensis*	JQ726581	Miehe 04-165-17	China,SE Tibet
Tbt2	*E.minuta*	JQ726582	Miehe et al. 98-35503	China,SE Tibet
Trk1	*E.distachya*	JQ726576	F. 29.772	Turkey, C Anatolia, Konya
Trk2	*E.distachya*	JQ726578	Nydegger 45.473	Turkey, E Anatolia
Trk3	*E.distachya*	JQ726583	H. Freitag 28.834	Turkey, Ankara prov., Tov Golu Lake
Tsc	*E.tihoana*	GU290485	H. Scholz 248(B)	Tschad,Tarso Tousside
Tuv	*E. pseudodistachya*	JQ726589	H. Freitag 33002	Russia: C Tuva
Ukr1	*E.distachya*	JQ726573	F. 33.259	Ukraine, S Crimea, Ayudag Mt.
Ukr2	*E.distachya*	JQ726574	F. 33.231	Ukraine, NE Crimea, Arabat
Uzb1	*E. distachya*	JQ726584	W. Wucherer 646	Uzbekistan: W coast Aral lake
Uzb2	*E.procera*	JQ726590	Freitag 33.121	Uzbekistan,Tashkent dist.

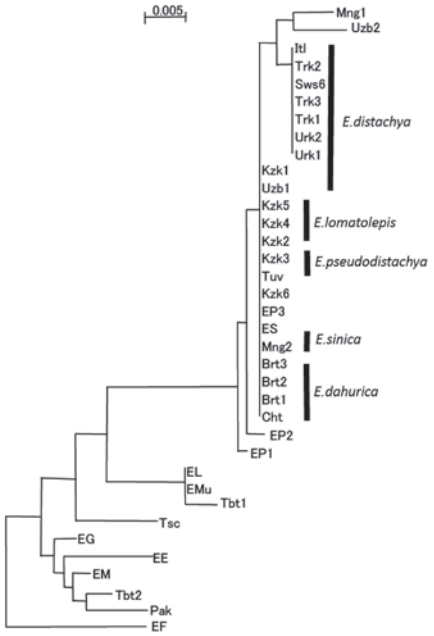
using ITS 2 sequences. We found that single nucleotide polymorphism in the ITS2 sequences, as the representatives are shown in Table 2. We pointed out the following evidences : (1) the sequence of *E. sinica* (ES) from China was almost identical to those of *E. dahurica* from the southern Buryatian region (Brt1 and Brt2) and Chita. (2) In Neighbor-Joining analysis of ITS2 (Fig 2), each of *E. pseudodistachya* and *E. lomatolepis* specimens from West to Central Asia form a cluster with *E. sinica* / *E. dahurica,* and (3) specimens of *E. distachya* from the Mediterranean Area to West Kazakhstan grouped together. The results confirmed the result obtained from ITS1 analysis as well as hypothesis derived from morphology that *E. sinica* and *E. dahurica* should be merged under the older name *E. dahurica*4).

Table 2 Single nucleotide polymorphism in ITS 2

	SNPs	nucletotide number in ITS2													
		16	17	19	49	84	93	169	205	218	220	230	243	246	247
References	*E. likiangensis* (EL)	C	C	G	C	G	G	G	A	C	A	G	A	A	A
	E. munuta (EMu)	*	*	*	*	*	*	*	*	*	*	*	*	*	*
	E. geradiana (EG)	*	*	*	*	*	*	*	*	*	*	T	G	*	*
	E. monosperma (EM)	*	*	*	*	*	*	*	G	*	*	T	G	*	*
	E. equisetina (EE)	Y	Y	*	*	*	A	*	R	*	*	T	G	*	*
	E. przewalskii (EP)	*	*	*	*	A	*	A	G	Y	G	*	*	G	*
	E. sinica (ES)	*	*	*	*	A	*	A	G	T	G	*	*	G	*
Samples	Brit 1	*	*	*	*	A	*	A	G	T	G	*	*	G	*
	Brit 2	*	*	*	*	A	*	A	G	T	G	*	*	G	*
	Tuv	*	*	*	*	A	*	A	G	T	G	*	*	G	C
	Kzk2	*	*	*	*	A	*	A	G	T	G	*	*	G	Y
	Trk1	*	*	*	T	A	*	A	G	T	G	*	*	G	*
	Trk2	*	*	*	T	A	*	A	G	T	G	*	*	G	*
	Ukr1	*	*	*	T	A	*	A	G	T	G	*	*	G	*
	Ukr2	*	*	*	T	A	*	A	G	T	G	*	*	G	*

As the reference, the following GenBank sequence data were used: *Ephedra equisetina* (EE): AY394065, *Ephedra gerardiana* (EG): AY394067, *Ephedra likiangensis* (EL): AY394068, *Ephedra monosperma* (EM): AY394066, *Ephedra minuta* (EMu): AY394069, *Ephedra przewalskii* (EP1): AY394064, *Ephedra sinica* (ES): AY394063. Asterisk (*) indicates that the respective nucleotide was the same as the top.

Fig 2 Neighbor - Joining analysis of ITS2 sequences.



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核リボソーム DNA，internal transcribed spacer 2 region (ITS2) 領域の塩基配列による *Ephedra sinica* 関連 *Ephedra* 属植物の系統解析

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要　旨

古来よりマオウ属植物は、日本及び中国で生薬として用いられており、中国の *Ephedra* 属植物の系統は 3 つの大きなグループに分かれている。現在、*Ephedra sinica* が日本及び中国市場で優位を占めている。*E. sinica* は、東および中央アジアに分布する *E. dahurica* や *E. pseudodistachya* と、形態的に類似している。一方西アジア及びヨーロッパに分布するマオウ属植物である *E. lomatolepis* や *E. distachya* との近縁性は、議論のあるところである。新たな生薬資源の探索の一環として、今回 *E. sinica* およびその近縁植物について、internal transcribed spacer 2 (ITS2) の塩基配列を分析および系統解析を行い、以下の結果を得た。（1）極東ロシア（ブリアチアおよびチタ）の *E. dahurica* 検体の ITS2 塩基配列は、中国及びモンゴル産 *E. sinica* とほぼ同一であり、のの 2 種は同種と考えられた。（2）中央アジア産の *E. pseudodistachya* と *E. lomatolepis* 検体は、*E. sinica* ／ *E. dahurica* と緩やかなクラスターを形成し、近縁性が示唆された。（3）西アジア及びヨーロッパ産の *E. distachya* 検体は、*E. sinica* ／ *E. dahurica* とは系統的に異なっていた。

キーワード：エフェドラ、系統、核リボソーム DNA、ユーラシア